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Enzymatic Transformations of Nicotine

Summary. Studies on the metabolism of nicotine in the dog have been extended to show that cotinine, in addition to being a urinary metabolite, is also an intermediary metabolite which can be demethylated, possibly by transmethylation or oxidative demethylation, to give desmethylcotinine. Cotinine is also hydroxylated to give hydroxycotinine which has been isolated from the urine as the picrate salt of the acetyl derivative.

The human subject metabolized nicotine to cotinine. The latter has been isolated from the urine of a volunteer nonsmoker after ingestion of nicotine. Cotinine also appears in the urine of smokers along with a variety of other metabolites. It is considered that the excretion of pyridine compounds by the smoker will present a problem more complex than that of the non-smoker in view of the incompleteness of knowledge on the chemical composition of smoke.

Studies on the synthesis of hydroxycotinine have been made. A number of compounds related to the two metabolites, cotinine and y-3-(pyridyl)-y-methylaminobutyric acid have been prepared. Preliminary evidence is given to indicate the feasibility of two synthetic routes to hydroxycotinine.

The production of cotinine in vitro by the hydrogen peroxide-catalase system points to the likelihood that cotinine can arise in vivo by enzymatic action which is dependent upon catalase and hydrogen peroxide.

INTRODUCTION

Our semi-annual progress report (July 1, 1957 - December 31, 1957) described the synthesis of a number of compounds of interest in nicotine metabolism, the isolation of y-3-pyridyl-y-methylaminobutyric acid (III) and cotinine (II) from urine after administration of nicotine (I) to the dog and establishment of the metabolic degradation of nicotine:

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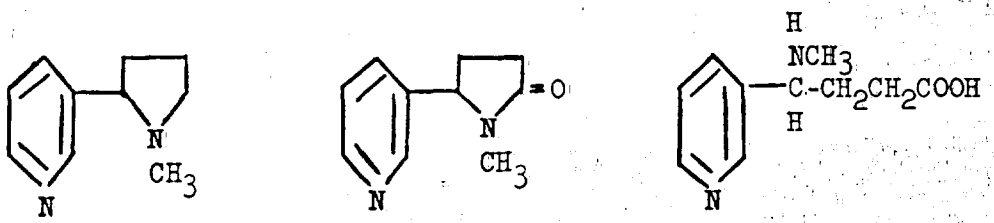

I II III

In addition to the foregoing preliminary data in support of the belief that cotinine played a key role in nicotine metabolism, pilot experiments on nicotine metabolism in the human were described.

The present report extends the observations which have previously been described, authenticates in part a hypothetical route of nicotine metabolism (cotinine ——> desmethylcotinine + hydroxycotinine), and provides an experimental basis for attributing to catalase an important enzymatic function in the degradation of nicotine in vivo.

For convenience of exposition this report is divided into the following sections:

I. Demethylation and Hydroxylation of Cotinine in vivo.

II. Metabolism of Nicotine in the Human.

III. The Urinary Excretion of Pyridine Compounds by Smokers.

IV. Hydrogen Peroxide-Catalase Oxidation of Nicotine.

V. Development of An Inexpensive Constant Rate Infusion Apparatus.

VI. Studies on The Synthesis of Derivatives of Cotinine.

VII. Publications and Papers Presented -- July 1, 1957-June 30, 1958.

VIII. Future Plans.

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II. METABOLISM OF NICOTINE IN THE HUMAN

Introduction

The previous semi-annual report describes preliminary studies in which a chloroform-extract of human urine was examined chromatographically

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and found to contain Koenig positive substances corresponding in Rf value to cotinine and "metabolite no. 3." These studies have been extended to facilitate a comparison with smokers' urine (section III) and unequivocal chemical identification of one of the metabolites present.

Experimental

A human subject (non-smoker) ingested 30 mg. of nicotine daily for three days in divided doses of 3 mg. per hour over a ten hour period on each day. The combined 24-hour samples of urine (total 5 liters) were extracted continuously with chloroform for twenty four hours. The residue from evaporation of the chloroform extract (567 mg.) upon paper chromatography (Whatman paper No. 1, ammonia, ethanol, butanol) yielded Koenig positive spots at Rf 0.61 and Rf 0.70. The former corresponded in Rf value to Koenig positive material obtained from urine of dogs following intravenous infusion of nicotine. The latter corresponded in Rf value to authentic cotinine. Upon chromatography (Whatman paper No. 1, sec-butanol, formic acid) Koenig positive spots at Rf 0.21 and 0.37 were obtained. Cochromatography showed the same correspondence with cotinine (Rf 0.37). This evidence seemed to suggest a resemblance between the metabolism of nicotine in the dog and in the human. In consequence chemical identification of cotinine was sought by an adaptation of the previously described methods. Control urine did not yield Koenig positive material.

The gummy material from the chloroform extraction was dissolved in 25 ml. of water and acidified to pH 2 with 5 N HCl. The solution was passed through a Dowex 50 (H^+) column. The column was washed with water until the effluent was neutral and then eluted with 80 ml. of N/1 ammonia water. The eluate was evaporated to dryness and then dissolved in 10 ml. of water and adjusted to pH 9 with N/1 ammonia water. The resulting solution was placed on a Dowex 1 ($O\bar{H}$) column (12 x 70 mm.). The effluent and water wash were combined and evaporated to dryness. The residue was treated with 25 ml. of chloroform and placed on a column of acid-washed alumina (5 g.) and eluted with ether containing 5-25 per cent methanol. The fractions yielding a single Koenig positive zone at Rf 0.72 (ammonia system) were combined and evaporated. The residue (10 mg.) of yellow oil yielded a crystalline picrate upon treatment with an excess of methanolic picric acid. The product was recrystallized to a constant micromelting point (100 - 102°) which was identical to that of an authentic sample of the picric acid salt of (-) cotinine. The mixed melting point showed no depression, and the infrared absorption spectra of authentic and metabolic products were identical.

Summary and Conclusion

Since normal urine of the non-smoker does not yield Koenig positive material when treated in the foregoing procedure it is established that cotinine arises from the metabolism of nicotine in the human.

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III. THE URINARY EXCRETION OF PYRIDINE COMPOUNDS BY SMOKERS

Pooled urine from male human smokers (60 l) was collected, in the presence of toluene or chloroform and sodium flouride as preservative, as voluntary daytime contributions. The urine was adjusted to pH 9-10 by an addition of ammonia water and then extracted continuously with chloroform. The residue from the chloroform extract (7.09 g.) was treated with 40 ml. of water. The aqueous solution was separated from a residue. The latter was treated with 40 ml. of methanol which was separated from a residue. That the latter was devoid of Koenig positive material was demonstrated by paper chromatography of a chloroform extract. The methanolic solution, which contained Koenig positive material, was evaporated to dryness. The residue was dissolved in the aqueous extract. The solution was acidified to pH 2 with HCl and placed on a Dowex 50 (H+) column (2.5 cm. x 15 cm.). The column was then washed with 700 ml. of water, and finally eluted with 500 ml. of N/1 ammonia water until all of the Koenig positive material was removed. The eluate contained Koenig positive material: Rf 0.61, 0.75, 0.85 and 0.90 (minor) when chromatographed in the ammonia-ethanol-butanol system. These zones corresponded in Rf value to zones present in the original aqueous and methanolic extracts of the chloroform extracts of urine.

The ammoniacal solution from the Dowex 50 column was passed through Dowex 1 ($O\bar{H}$) (2.5 x 15 cm.). The effluent and water wash (1 l.) was concentrated to a yellow syrup (1.1 g.). The column was then eluted with 200 ml. of 1 m acetic acid. The acetic acid solution was concentrated to a brown syrup (459 mg.). The latter was dissolved in 20 ml. of absolute ethanol and treated with decolorizing carbon. The filtrate was evaporated to dryness. The brown residue (284 mg.) which showed a single Koenig positive zone at Rf 0.73 (ammonia-ethanol-butanol) was saved for further investigation.

The basic material (1. 1 g.) which was collected as effluent from the Dowex 1 ($O\bar{H}$) was dissolved in 25 ml. of chloroform and placed on a column of alumina (30 g.). The column was eluted with ether and methanolic ether. The fractions from the 0-10% methanolic ether contained Koenig positive material at Rf 0.71 and Rf 0.77 (ammonia-ethanol-butanol). A further fraction of Koenig positive material at Rf 0.61 was also obtained.

Those fractions with Rf 0.71 and Rf 0.77 concentrated to yield 668.5 mg. of white solid. By rechromatography with 0-5% methanolic ether the two concentrates were separated to give 150 mg. of crystalline material with Rf 0.77 and λ max 272 mu in ethanolic HCl. This will receive further study.

The fractions containing the Koenig positive material at Rf 0.71 were combined and concentrated to a brown gum (87 mg.). By cochromatography

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with cotinine it was inferred that cotinine was present. Confirmation was achieved by conversion to cotinine picrate (m.p. 100-102°) which did not depress the melting point of authentic material.

The fractions with Rf 0.61 upon concentration yielded 118 mg. of brown gum. The material cochromatographed on paper with the desmethylcotinine-"hydroxycotinine" fraction obtained from nicotine metabolism in dogs. In consequence this material was treated with acetic anhydride and pyridine. The residue from concentration of this mixture was treated with methanol and reconcentrated to give 143 mg. of brown oil. This oil was dissolved in 5 ml. of chloroform and placed on a column of alumina (5 g.). The column was developed with methanolic ether (0-100%). To give Koenig positive material at Rf 0.75 and Rf 0.64 which were further investigated.

The Rf 0.75 fraction (80 mg.) consisted of an oil which was chromatographically identical on paper with acetylated hydroxycotinine which was previously isolated from the urine of dogs after nicotine infusion.

The Rf 0.64 fraction (17 mg.) was chromatographically identical with desmethylcotinine.

In addition to the foregoing survey of chloroform-soluble constituents of smokers' urine a preliminary survey of the substances not soluble in chloroform has been conducted. Both the aqueous and chloroform fractions present an experimental situation which is far more complicated than the corresponding situation observed following administration of nicotine to either the human or the dog.

For the moment no single explanation may be offered for the observed differences between the excretion of Koenig positive materials by the smoker and nicotine-treated subject. It is reasonable to suppose that some of the differences may arise as a result of absorption by the smoker of pyridine compounds other than nicotine. These substances may be metabolized to give a considerable variety of compounds. A further possibility is that the additional pyridine compounds may inhibit nicotine metabolism at some stage and thus give rise to new excretion products. Some of these factors will be considered in future studies.

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IV. HYDROGEN PEROXIDE-CATALASE OXIDATION OF NICOTINE

During the past few years emphasis has been given to methods for the isolation, characterization and identification of metabolites of nicotine. This work has made possible current investigations in which the physiological activity of the metabolites are being studied. In addition, the

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variety of nicotine metabolites now available makes possible both theoretical and applied studies of the role of specific enzymes in the production of metabolites.

Present and previous reports present data which favors the belief that cotinine plays a key role in the metabolism of nicotine. Pilot experiments have, therefore, been conducted to establish the possible enzymatic route to cotinine.

Since cotinine has been observed as the result of autoxication of nicotine, it was considered that the formation of cotinine in vivo might arise as a result of the action of hydrogen peroxide-catalase action on nicotine. Keilin and Hartree have reported that ethanol is oxidized to acetaldehyde in vitro in the presence of hydrogen peroxide in phosphate buffer and that yields of the acetaldehyde are enhanced when the reaction is carried out in the presence of catalase. In our studies nicotine was treated with hydrogen peroxide and catalase under conditions similar to those employed by Keilin and Hartree for ethanol. Following the oxidative procedure the reaction mixture was steam distilled under alkaline conditions to remove unreacted nicotine. Aliquots of the solution were then extracted with chloroform. The chloroform solution was chromatographed on Whatman No. 1 with ammonia-ethanol-butanol. The single Koenig positive zone on the chromatogram corresponded in Rf value to that obtained from an authentic sample of cotinine. This experiment does not unequivocally establish the involvement of catalase in the oxidation of nicotine in vivo. It does provide, however, material evidence on which to develop further experimental studies.

V. DEVELOPMENT OF AN INEXPENSIVE CONSTANT RATE INFUSION APPARATUS

Introduction

In a variety of pharmacological and chemical studies there is a requirement for a device which will permit the infusion of solutions at a constant rate to an organism or chemical mixture. In simple chemical reactions there are a variety of devices for proportionate feed or constant rate injection of solutions can be accomplished by manual means, which are often tedious and inaccurate, or with the aid of positive drive mechanisms which are generally expensive, although under ideal conditions trouble-free and accurate. Apparatus in the latter category is usually elaborate and, in cases where simultaneous multiple tests are conducted, the cost of the apparatus can well exceed several thousand dollars.

In the course of studies in this laboratory where dogs were given aqueous solutions of nicotine intravenously over an eight-hour period it was desirable to develop moderately-priced apparatus for simultaneous

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infusion at constant rate to six or more animals. By the advantageous selection of inexpensive commercial components such an apparatus was developed. So far as we are aware, no equivalent device has been described. The wide range of applicability of this simple apparatus prompts a report of it.

Experimental and Discussion

Dogs under pentobarbital anesthesia were cannulated at the femoral vein. The cannulae were attached by polyethylene tubing to a 25 or 50 ml. burette, equipped with solenoid valve (Houston Glass Fabricating Company, 5313 Harrison Boulevard, P.O. Box 9032, Houston 11, Texas, Cat. No. 824-2). The burette was enlarged at the top by means of a 60 mm. powder funnel which was connected by a short length of tubing to the top of the burette. In trial runs for adjustment a non-toxic solution such as saline was used to fill the burette. The solenoid valve was actuated by means of a repeat cycle timer (Andrew Technical Supply, 6972 North Clark Street, Chicago 26, Illinois). For the purposes of the study a delivery of one ml. in each 60 second period was selected. The timer (normally open) was set to close for one second in each minute.

After initial adjustment by a screw clamp to the required rate of flow, which is usually easily achieved empirically within the first 5 ml. of the burette by observing the initial rate and subsequent loss of rate (approximately 0.1 ml. with decrease of hydrostatic head corresponding to -1 ml. on the burette), the burette and reservoir are filled and positioned. The apparatus will infuse solutions intravenously to individual animals over 80 to 325 minute periods with an accuracy in the range of 10 per cent. Venous pressure is sufficiently constant to allow even flow from the time of the initial adjustment.

Since the apparatus is all glass, with the exception of the indicated organic material which can be readily converted to glass, corrosion does not become a problem. Tolerances are wide enough to insure ready assembly from ordinary parts, but some attention must be given to the diameter and wettability of the tube leading from the reservoir into the funnel to insure constant filling of the funnel.

If attention is given to stable placement of the cannula and elimination of foreign particles from the solutions no difficulties are ordinarily encountered. Grit and large particulate matter if present will prevent the solenoid valve from seating properly, and bring about an early emptying of the reservoir.

The total cost of special components for one animal is approximately forty dollars. Six or more additional animals may be simultaneously

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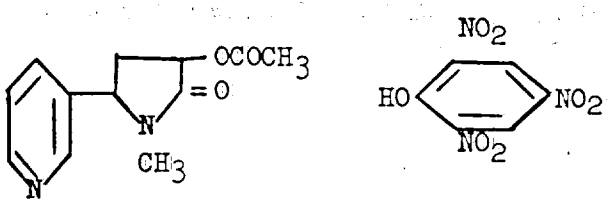
infused by the same timer at an approximate cost of fourteen dollars each. Additional economy can be effected by use of a surplus one r.p.m. motor mounted to actuate a microswitch by means of a heart cam or cam wheel.

Summary

An apparatus for infusion at constant rate, readily assembled from inexpensive commercial components has been described.

VI. STUDIES ON THE SYNTHESIS OF DERIVATIVES OF COTININE

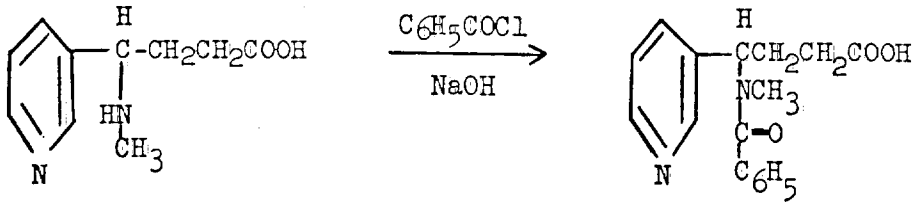
Following the administration of cotinine the urine of dogs contains a substance which, after chromatographic partial purification, can be acetylated with acetic anhydride-pyridine to yield an oil. The latter forms a crystalline picrate which has an empirical formula consistent with the following structural formula:



Preliminary data is sufficient to suggest that the metabolite "hydroxy-cotinine" has an OH group which is α to the carbonyl group of the pyrrolidone ring.

Based on the foregoing, several synthetic routes to the metabolic compound have been explored. Two routes now show promise and are now summarized to indicate the steps which have been carried out (a more detailed report will follow):

1.



(I) (II)

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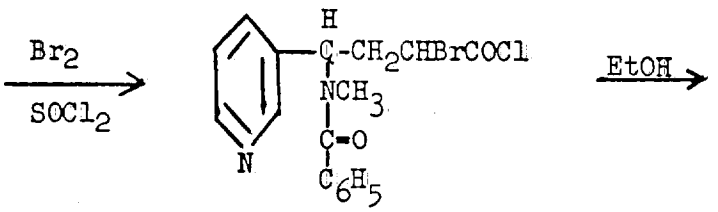

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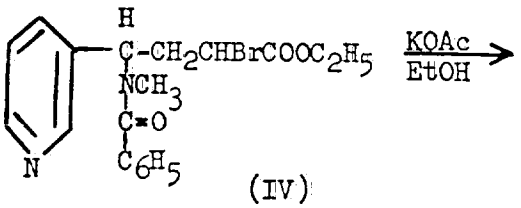

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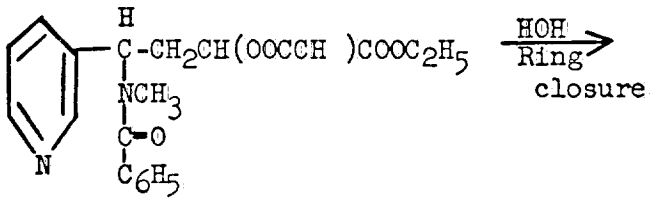

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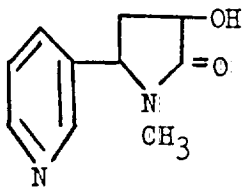
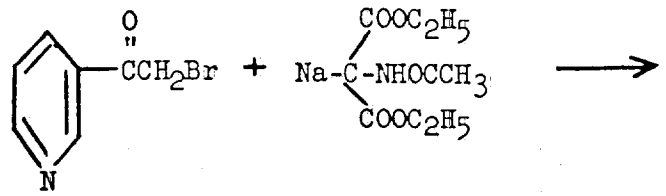


(VI)

2.



(I) (II)

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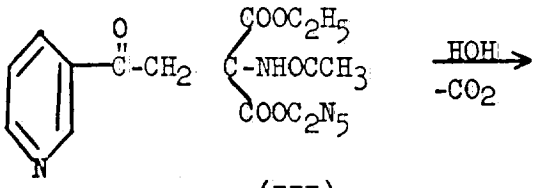

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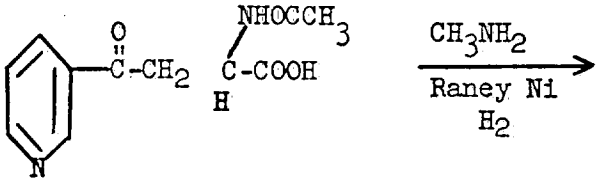

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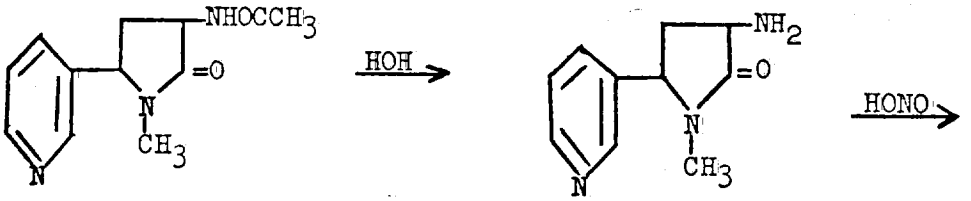

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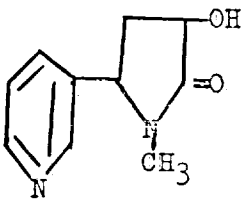

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In synthesis 1 intermediate (II) was prepared in good yield. The subsequent steps have yielded compounds which although not isolated in pure form appeared to be adequately pure to carry out the sequence. The final product (VI) hydroxycotinine although impure had chromatographic properties consistent with natural metabolic "hydroxycotinine."

In synthesis 2 products through (VI) have been obtained in good yield. Analytical data is not yet complete. Treatment of "aminocotinine" (VI) with nitrous acid afforded a product which cochromatographed with natural "hydroxycotinine."

Since both of the foregoing syntheses might be expected to yield a mixture of diastereoisomers in contrast to the single isomeric form expected in metabolism, steps are now being taken to prepare sufficient quantities of the materials to effect a separation of isomers for further chemical and biological studies.

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VII. PUBLICATIONS AND PAPERS -- JULY 1, 1957 - June 30, 1958

Publications

1) Synthesis and Properties of Pyridylalanines, Herbert McKennis, Jr., and Edward R. Bowman, The Virginia Journal of Science 8, 314 (1957).

2) Gamma-(3-Pyridyl)-Gamma-Methylaminobutyric Acid as a Urinary Metabolite of Nicotine, Herbert McKennis, Jr., Lennox B. Turnbull and Edward R. Bowman, Journal of the American Chemical Society 79, 6342 (1957).

3) Metabolites of Nicotine and a Synthesis of Normicotine, Herbert McKennis, Jr., Lennox B. Turnbull, Harvey N. Wingfield, Jr., and Lovell J. Dewey, Journal of the American Chemical Society 80, 1634 (1958).

4) Metabolism of y-(3-Pyridyl)-y-Oxobutyric Acid, Lennox B. Turnbull, Edward R. Bowman and Herbert McKennis, Jr., Federation Proceedings 17, 421 (1958).

Papers Presented

1) Metabolic Fate of the Pyrollidine Ring of Nicotine, Herbert McKennis, Jr., Lennox B. Turnbull, and Edward R. Bowman, Southeastern Regional Meeting, American Chemical Society, Durham, N.C., Nov. 14, 1957.

2) New Metabolites of Nicotine, Lennox B. Turnbull, Herbert McKennis, Jr., and Edward R. Bowman, Biological Seminar, Medical College of Virginia, March 5, 1958.

3) Metabolism of y-(3-Pyridyl)-y-Oxobutyric Acid, Lennox B. Turnbull, Edward R. Bowman, and Herbert McKennis, Jr., 48th Annual Meeting, American Society for Pharmacology and Experimental Therapeutics, Philadelphia, Penna., April 16, 1958.

4) Metabolism of Nicotine in the Human, Edward R. Bowman, Lennox B. Turnbull, and Herbert McKennis, Jr., Annual Meeting, The Virginia Academy of Science, Roanoke, Va., May 9, 1958.

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VIII. Future Plans

A continuation of studies on the synthesis of hydroxycotinine and related compounds is contemplated. This will facilitate chemical characterization of urinary metabolites and aid in the interpretation of enzymatic studies on the degradation of nicotine. A parallel study of nornicotine metabolism is being inaugurated to facilitate interpretation of the urinary pyridine compounds of the smoker and to assist in the interpretation of nicotine metabolism.

Since the current program is now entering the final year of the three year period under support of the Tobacco Industry Research Committee it is planned to formulate a reapplication for support in the coming fall.

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